INFECTION AND IMMUNITY, Sept. 2010, p. 3905–3919 Vol. 78, No. 9
0019-9567/10/$12.00 doi:10.1128/IAI.00344-10

E3 Ubiquitin Ligase Activity and Targeting of BAT3 by Multiple *Legionella pneumophila* Translocated Substrates[▽]

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Received 6 April 2010/Returned for modification 6 May 2010/Accepted 4 June 2010

The intracellular bacterial pathogen *Legionella pneumophila* modulates a number of host processes during intracellular growth, including the eukaryotic ubiquitination machinery, which dictates the stability, activity, and/or localization of a large number of proteins. A number of *L. pneumophila* proteins contain eukaryotic-like motifs typically associated with ubiquitination. Central among these is a family of five F-box-domain-containing proteins of *Legionella pneumophila*. Each of these five proteins is translocated to the host cytosol by the Dot/Icm type IV protein translocation system during infection. We show that three of these proteins, LegU1, LegAU13, and LicA, interact with components of the host ubiquitination machinery *in vivo*. In addition, LegU1 and LegAU13 are integrated into functional Skp–Cullin–F-box (SCF) complexes that confer E3 ubiquitin ligase activity. LegU1 specifically interacts with and can direct the ubiquitination of the host chaperone protein BAT3. In a screen for additional *L. pneumophila* proteins that associate with LegU1 in mammalian cells, we identified the bacterial protein Lpg2160. We demonstrate that Lpg2160 also associates with BAT3 independently of LegU1. We show that Lpg2160 is a translocated substrate of the Dot/Icm system and contains a C-terminal translocation signal. We propose a model in which LegU1 and Lpg2160 may function redundantly or in concert to modulate BAT3 activity during the course of infection.

Legionella pneumophila is a Gram-negative bacterial pathogen with a facultative intracellular lifestyle in a strikingly broad range of host cells (30). In both the natural host, amoebae, and its accidental host, mammalian macrophages, the intracellular replication of *Legionella* within a nonacidified vacuole depends largely on the Dot/Icm type IVB secretion system (60, 68). This system translocates a very large repertoire of bacterial substrates into the host cytosol, whose functions are thought to be largely redundant during intracellular replication in host cells (28, 51). These substrates target host cell secretory trafficking, translation, apoptosis, and other processes in support of bacterial growth (34).

One of the conserved host pathways that *Legionella pneumophila* impinges on is the ubiquitin-mediated proteasomal degradation pathway. Knockdown experiments have demonstrated that a host chaperone of ubiquitinated proteins, VCP/Cdc48, contributes to *Legionella* replication within insect and mammalian cells (25). Notably, polyubiquitinated species accumulate around the *Legionella*-containing vacuole (LCV) shortly after uptake into the host cell (25, 35, 53). This polyubiquitination may play a role in the intracellular trafficking of translocated substrates, as VCP mediates the removal of individual substrates from the vacuolar surface (25). A number of *Legionella* genes contain domains that implicate them as possible modulators of the host ubiquitination system (15, 16, 21). Chief among these potential effectors are those with predicted E3 ligase activity, as such activity might direct the ubiquitination of specific host or bacterial targets during infection (14).

The primary purpose of a functional Skp–Cullin–F-box (SCF) E3 ubiquitin ligase is to facilitate the transfer of ubiquitin from the ubiquitin conjugating enzyme (E2) to a target substrate (70). In an SCF complex, a Cullin protein, usually CUL1, forms a scaffold upon which the rest of the complex is built (Fig. 1A). RBX1, a RING-box protein, associates with both CUL1 and the ubiquitin conjugating enzyme (E2). SKP1 functions to link this enzymatic part of the complex to an F-box protein. F-box proteins, in turn, act as substrate specificity modules of the SCF via a compact, modular structure that facilitates mimicry by a wide range of pathogens (2). F-box proteins bind to the SCF complex through the F-box domain, a small Skp1-binding domain at the N terminus. C-terminal sequences confer substrate specificity to the F-box protein, directing the complex to ubiquitinate proteins that specifically interact with these sequences. The effector repertoire of some bacterial and viral pathogens includes F-box proteins that, once they are delivered to the host cell, co-opt the eukaryotic ubiquitination machinery to target other host and/or bacterial proteins for degradation (2).

One of the first bacterial F-box proteins identified was VirF, from the plant pathogen *Agrobacterium tumefaciens* (59). VirF has been shown to target both a host protein (VIP1) and a bacterial protein (VirE2) for ubiquitination and proteasomal degradation (67). The plant viral pathogen polerovirus also encodes an F-box protein (P0) that targets host Argonaute 1 for ubiquitination and degradation (7). Notably, the recently described genome of the amoebal symbiont *Amoebophilus asiaticus* encodes several proteins that may interfere with the host ubiquitination system, including 15 proteins with predicted F-box motifs and 9 proteins with E3 ligase-associated U-box domains (58). U-box proteins are E3 ligases that, unlike F-box proteins, do not associate with other proteins in order to form

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▽ Published ahead of print on 14 June 2010.

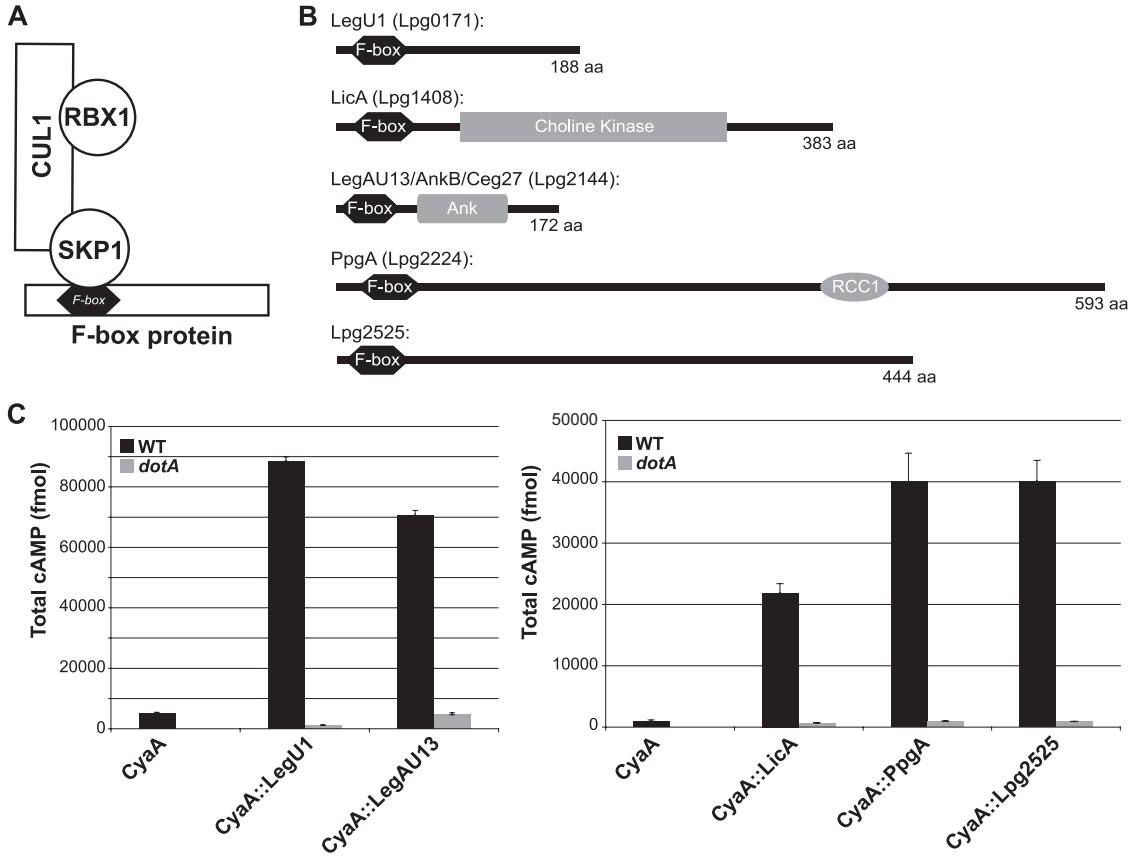

FIG. 1. *L. pneumophila* contains several Dot/Icm translocated substrates with F-box domains. (A) Structure of the SCF complex. An F-box-domain-containing protein associates with SKP1. SKP1 and RBX1 associate with CUL1, which serves as a scaffold for complex formation. (B) Five proteins contain predicted F-box motifs in the *Legionella pneumophila* strain Philadelphia-1 genome. aa, amino acids; Ank, ankyrin repeat domain; RCC1, regulation of chromatin condensation domain. (C) Each of the *L. pneumophila* F-box proteins is translocated into the host cytosol in a Dot/Icm-dependent manner. U937 cells were challenged with wild-type or translocation-deficient *dotA* bacteria expressing CyaA (negative control) or CyaA::LegU1, CyaA::LegAU13, CyaA::LicA, CyaA::PpgA, or Cya::Lpg2525. After 1 h, host cells were lysed and cAMP levels were quantified. The data represented are the averages of three replicates ± standard errors of the means.

an E3 ligase complex (31, 36). U-box-containing effectors have also been described for the plant pathogen *Pseudomonas syringae*, and these effectors prevent host cell death by ubiquitinating the host kinase, Fen (55). Similarly, the *L. pneumophila* LegU2/LubX protein contains a U-box domain and has been shown to ubiquitinate the host protein Clk1 (37).

All strains of *L. pneumophila* sequenced to date encode multiple genes with predicted F-box motifs, including five F-box genes in *L. pneumophila* strain Philadelphia-1 (2, 16, 21). One of the F-box proteins, LegAU13, has been shown to associate with SKP1 in mammalian cells, but it has not been shown to associate with other components of the SCF complex (40, 53). None of the predicted *L. pneumophila* F-box proteins, including LegAU13, have been shown to act as functional E3 ubiquitin ligases capable of directing the polyubiquitination of target proteins.

In the Philadelphia-1 strain, single mutants harboring in-frame deletions of each of three of the five *L. pneumophila* F-box genes, *legU1*, *legAU13*, and *licA*, do not display defects in the accumulation of polyubiquitinated species to the LCV during intracellular replication (35). Furthermore, no defect in bulk ubiquitination was observed for a quadruple mutant strain lacking *legU1*, *legAU13*, *licA*, and the U-box gene *legU2* (35). In the AA100 and Paris strains of *L. pneumophila*, others have observed different results (1, 40, 52, 53). After uptake into host cells, ubiquitinated species do not accumulate around the LCV of AA100 bacteria harboring a single insertional mutation in the *legAU13*/*ankB* gene (52, 53). The same strain also displayed an intracellular growth defect in mammalian and amoebal hosts (1, 53). *L. pneumophila* Paris bacteria harboring an in-frame deletion of the *legAU13* ortholog, Lpp2082, are also severely defective for ubiquitin recruitment to the LCV (40). However, deletion of *legAU13* in Paris has a much more modest impact on intracellular replication than in the AA100 insertional mutant (1, 40). The inconsistency between the observed phenotypes of *legAU13* mutant strains may be due to differences in strain backgrounds.

We demonstrate here that three of the F-box-motif-containing proteins (LegU1, LegAU13, and LicA) associate with multiple components of the host ubiquitination machinery. For the first time, we show that LegU1 and LegAU13 confer an E3 ligase activity and can direct polyubiquitination *in vitro*. Furthermore, we show that the targets of SCF^{LegU1}-mediated ubiquitination include the broadly functioning host protein

BAT3, a cochaperone of Hsp70 (19) that is involved in the regulation of the endoplasmic reticulum (ER) stress response (24). In addition, we identify a Dot/Icm translocated substrate, Lpg2160, that also interacts with BAT3 in mammalian cells. Taken together, these data support a model in which *Legionella* uses multiple effectors to co-opt the host ubiquitination machinery and to modify the activity of BAT3 during replication within the eukaryotic host.

MATERIALS AND METHODS

Bacterial growth and transgenics. All *Legionella* strains used in this study were derived from a clinical isolate of *Legionella pneumophila*, strain Philadelphia-1 (8). Restriction-defective strain Lp02 (*thyA rpsL* Δ*lvh*) was cultivated on charcoal–*N*-(2-acetamido)-2-aminoethanesulfonic acid (ACES)–yeast extract–thymidine (CYET) plates and in ACES-yeast extract-thymidine (AYET) broth (32). Plasmids were introduced by electroporation (9) or by mating with *Escherichia coli* Tra^+ helper strain RK600 (64). In-frame deletion mutations were generated using derivatives of the suicide vector pSR47S (26). Challenge of mammalian cells with *L. pneumophila* was performed as described previously (32), using bacteria grown overnight to post-exponential phase, which are predominantly motile (A_{600} = 3.7 to 4.5). Growth curve studies for the bacteria within mammalian cells were performed as described previously at an initial multiplicity of infection (MOI) of 0.01 (32). To facilitate amoebal challenge, the thymidine auxotrophy of Lp02-derived *L. pneumophila* strains was rescued by natural transformation with a plasmid containing a wild-type copy of the *L. pneumophila* $thyA^+$ open reading frame, pJB3395 (a kind gift from J. P. Vogel). Briefly, each strain was patched on CYET plates and incubated at 30°C overnight. On the next day, 800 ng of plasmid was added to each patch on the plate, and the components were mixed gently and returned to 30°C for an additional overnight incubation. As this plasmid does not replicate in *L. pneumophila*, a double crossover replaces the *thyA* mutant allele of each strain with a wild-type copy. Thy^+ transformants were selected for by streaking these transformed bacteria onto CYE plates (lacking thymidine). Amoebal growth curves were performed using *Acanthamoeba castellanii* grown in peptone-yeast extract-glucose (PYG) broth (47) at 25°C to 90 to 100% confluence in T175 tissue culture flasks. Monolayers of amoebae were harvested by incubation with ice-cold phosphate-buffered saline (PBS) for 5 to 10 min, and aliquots of 2.5×10^7 amoebae/ml were slowly frozen to −80°C in 10% dimethyl sulfoxide (DMSO)–90% fetal bovine serum (FBS). Amoebae were thawed prior to use, resuspended in *A. castellanii* buffer (47), and transferred in 100-μl aliquots of 5×10^5 amoebae to each well of a 96-well tissue culture plate; and the plate was incubated overnight at 37°C. Challenge of these amoebae on the next day was performed as described above, at an MOI of 0.01. One hour after challenge, the *A. castellanii* buffer in each well was removed and replaced with 100 μl of fresh *A. castellanii* buffer. Bacteria were harvested at each time point by transferring the contents of each well to a fresh plate and lysing the remaining monolayer of amoebae with 100 μl of sterile H_2O–0.05% saponin for 10 min at 37°C. For each well, these two 100-μl samples were combined with each other, along with a final 100 μl of the wash of each well with H_2O–0.05% saponin, for a total of 300 μl of sample per well. The numbers of CFU were determined by plating a series of dilutions onto CYE plates and quantifying the number of colonies 3 to 4 days later.

Plasmids. A plasmid encoding the BAT3::Myc-tagged protein was a kind gift from S. Kornbluth (57). The mammalian HA::ubiquitin expression plasmid pMT123 and derivatives were kind gifts from D. Bohmann and S. Lippard (65). SKP1, RBX1, and CUL1 were individually cloned into pcDNA3.1/Hygro(+) (Invitrogen) by PCR amplification of each gene from HEK-293T cDNA with PfuUltraII polymerase (Stratagene) with the following primers: hCUL1F (CGG GATCCTGCTGCACTGGACGACTTTA), hCUL1R (TACGTCCTGCGGCC GCAACTATTTGCTGCGGGTCAC), hSKP1F (CGGGATCCCCGTCTCCTT AACACCGAAC), hSKP1R (TACGTCCTGCGGCCGCGACTTGCTGCTGC ATTTGTC), hRBX1F (CGGGATCCACCGTGTGTTTCCAAAATGG), and hRBX1R (TACGTCCTGCGGCCGCAGGTAACAGCAGGGAAAGTCA). The complete open reading frames of the *legU1*, *legAU13*, *licA*, *ppgA*, and *lpg2525* genes were first cloned into pcDNA3.1/Hygro(+) by PCR, and then the 3XFLAG epitope was inserted upstream of each via subcloning of a PCR fragment generated by amplification of the 3XFLAG plasmid pEY221 (a kind gift from E. Yigit and C. Mello). Green fluorescent protein (GFP)-tagged versions of *lpg2160* and *lpg2638* were generated as described previously (41). An HA::mYFP (where YFP is yellow fluorescent protein) plasmid based on the pEGFP-C1 backbone (69) was used to clone *lpg2160* for anti-HA immunoprecipitation studies. The primers used were 3XFLAG_F (TTGTTGCAAGCTTGCAATGG CCGGCCGCGACTATAAGGACGA), 3XFLAG_R (AATTCGGATCCGGC GGCCGCTTTGTCATCATC), lpg0171_ORF_F (GCGCGCGGATCCATGA AAGCAAAATACGAC), lpg0171_ORF_R (GCAGCTGCGGCCGCGTTACA ATGGCTCACAT), lpg0171ΔFbox_invF (TTGGTACCTGTGGGGTCGTATT TTGCTT), lpg0171ΔFbox_invR (CCGGTACCACGAATACATTATTTCGTC TTGATAA), lpg1408F_ORF_F (GCGGATCCATGACGTTTATGAAATTAT ATTTTTTTGGTG), lpg1408R_ORF_R (TCATATGCGGCCGCTAGATAAT GGCGGCAGCTAA), lpg1408ΔFbox_invF (TTGGTACCTACATCACCAAA AAAATATAATTTCATAAACG), lpg1408ΔFbox_invR (CCGGTACCGTGA TTCATCGAATACCTTTATTTGC), lpg2144_ORF_F (GCGCGCGGCCGGA TCCATGAAAAAGAATTTTTTTTCTG), lpg2144_ORF_R (GCGCGGCGC GCGGCCGCGTTAACAAACAAGGCAC), lpg2144ΔFbox_invF (TTGGTAC CTTTCATGGATCCGGCG), lpg2144ΔFbox_invR (CCGGTACCAGACAAC AGCATATAAAGCGAGAG), lpg2224_ORF_F (GCGGATCCATGAAAGA ACCTCGTGAACTTAATC), lpg2224_ORF_R (TCATATGCGGCCGCATGT TTTGCTTAGGCGCTTC), lpg2525_ORF_F: (GCGGATCCATGAAAGAAC CTCGTGAACTTAATC), lpg2525_ORF_R: (TCATATGCGGCCGCATGTT TTGCTTAGGCGCTTC), lpg2160_HAYFP_F (AGAATTCTCTGTTCTCAG CTAATCAATCTCTAGATT), lpg2160_HAYFP_R, and (TTGGTACCTTAC ATAGGACTTGCAACACTCCTG). The CyaA fusion plasmid used for the translocation studies has been described previously (32). Full-length opening reading frames of each F-box gene were introduced into the CyaA fusion plasmid by subcloning from 3XFLAG-tagged mammalian expression plasmids. PCR products of full-length *lpg2160*, a C-terminal fragment, and a C-terminal deletion were cloned into the CyaA plasmid. The primers used in the generation of each CyaA::Lpg2160 fusion plasmid were as follows: lpg2160_cya_F (TGTTGCGTC GACCTGTTCTCAGCTAATCAATCTCTAGATT), lpg2160_cya_R (TGTTG CGCATGCTTACATAGGACTTGCAACACTCCTG), lpg2160_cya_dC_R (TGT TGCGCATGCTTAATTTCGTCCAATAATCTTACAAAGTTTC), and lpg2160_ cya_Conly_F (TGTTGCGTCGACCGAAATTATCTGGATCCTTCGT).

Mammalian cell culture and transfection. Primary bone marrow-derived macrophages from female A/J mice were isolated as described previously (4), frozen in 10% DMSO–90% fetal bovine serum, and thawed prior to use. Primary bone marrow macrophages and U937 cells were grown in RPMI–10% heat-inactivated FBS at 37°C with 5% CO_2. U937 cells were differentiated into adherent phagocytes using 12-*O*-tetradecanoylphorbol-13-acetate (TPA), as described previously (8). HEK-293T cells were grown in Dulbecco modified Eagle medium–10% heat-inactivated FBS. For transfection, HEK-293T cells were seeded at 6×10^6 cells in 10-cm tissue culture treated dishes and incubated overnight. Plasmids were transfected using Lipofectamine 2000 (Invitrogen), following the manufacturer's suggested procedure for 60 μl Lipofectamine 2000, and 24 μg plasmid DNA. Transfections using multiple plasmids were performed under these conditions, but the total DNA amounts were adjusted to 24 μg for each 10-cm dish of cells. Pools of GFP-tagged putative substrates and 3XFLAG-tagged LegU1ΔF-box were transfected using 2 μg per plasmid at a ratio of 23:1 (GFP-fusion plasmids/3XFLAG::LegU1ΔF-box plasmid). 3XFLAG-tagged LegU1 lacking the F-box domain was used for these analyses to stabilize any possible associations that might be disrupted by the E3 ligase activity of full-length LegU1.

Measuring translocation of putative substrates. Translocation of putative Dot/Icm substrates into target cultured cells was measured using the adenylate cyclase fusion assay, as described previously (32). Briefly, differentiated U937 cells were challenged with wild-type or translocation-deficient (*dotA*) *L. pneumophila* for 1 h at an MOI of 1. The cells were lysed, and intracellular cyclic AMP (cAMP) levels were measured using a cAMP Direct Biotrak enzyme immunoassay kit (GE Healthcare Life Sciences).

qRT-PCR of *Legionella* transcripts. Total RNA was purified using an RNeasy mini (Qiagen) kit, as directed, starting with cultures of wild-type *L. pneumophila* (Lp02) grown in AYET medium. RNA was DNase treated using a Turbo DNAfree kit (Ambion), and 1 μg of total RNA was reverse transcribed into cDNA using Superscript III (Invitrogen) and random decamer primers (Ambion). Defined dilutions of Lp02 genomic DNA were used as templates in quantitative PCRs (qPCRs) to derive a standard curve, and diluted cDNAs were used as experimental templates in quantitative reverse transcription-PCRs (qRT-PCRs) using a Stratagene Mx3005P real-time PCR machine. The standard deviations (SDs) of the qRT-PCR values for the levels of the tested gene (*X*) normalized to 16S rRNA (16S) levels were calculated using the following formula: SD of (*X*/16S) = (quantity *X*/quantity of 16S) · {square root of [(SD of *X*/quantity of *X*)^2 + (SD of 16S/quantity of 16S)^2]}) The primers were as follows: 16S_sdsF (CTAAGGAGACTGCCGGTGAC), 16S_sdsR (CGTAAGGGCCAT GATGACTT), lpg0171_sdsF (ATAACCTCTCTCGCCCATGA), lpg0171_sdsR (TGCATGCTGTTTCTCAGCTT), lpg1408_sdsF (CGCTAAACAAGCCGAA

AAAC), lpg1408_sdsR (CGTTACCTGCAACCCATCTT), Lpg2144_sdsF (ACTGGCAAATCCAGCCTTC), lpg2144_sdsR (CCCCTAATTAAGGCGCGTAT), lpg2224_sdsF (GGGGTTCCAAGCAAGAAACT), lpg2224_sdsR (GCAATGTGAGAATGGGTGTG), lpg2525_sdsF (GATGGGGATGGAATTCATTG), lpg2525_sdsR (ATATCGCGGCCATTTGTATC), sidC_sdsF (AGGCAAACATCCTCTTGGTG), and sidC_sdsR (TGTAAGCGATTGGAGGTTCC).

Immunoprecipitation and Western blot analysis. HEK-293T cells were transfected with mammalian expression plasmids, and lysates were harvested 18 to 24 h after transfection, as follows: after removal of the growth medium, 1 ml of lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) was added directly to the cells, which were subsequently incubated at 4°C for 20 to 30 min. Lysates were transferred to microcentrifuge tubes, and the debris was pelleted by centrifugation at >14,000 × *g* for 15 min at 4°C. The cleared supernatants were then transferred to new microcentrifuge tubes, taking care to not disturb the pellet. The concentration of protein in each cleared supernatant was quantified using the Bradford assay (Bio-Rad). Each sample was diluted in additional lysis buffer to adjust each sample so that it had an equal concentration of protein in 1 ml of total lysis buffer (typically, 1 mg/ml). Immunoprecipitation was performed using anti-FLAG M2 affinity gel (Sigma), EZview Red antihemagglutinin (anti-HA) affinity gel (Sigma), and an anti-c-Myc agarose affinity gel (Sigma). For each sample, 40 μl of resin was pelleted at 8,000 × *g* for 30 s, washed in 1 ml ice-cold Tris-buffered saline (TBS; 50 mM Tris, 138 mM NaCl, 2.7 mM KCl, pH 8.0), pelleted again, and then resuspended in the lysate. After incubation overnight at 4°C on a tube rotator, resin was collected by gentle centrifugation (8,000 × *g*, 30 s, 4°C) and washed two to three times with 1 ml ice-cold lysis buffer and three times with 1 ml ice-cold TBS. Immunoprecipitates were released from the resin by boiling in sample buffer and were loaded on SDS-polyacrylamide gels for analysis by immunoblotting. The antibodies used for immunoblotting (and their dilutions) were as follows: rabbit anti-SKP1 (1:1,000; Abcam), rabbit anti-CUL1 (1:500; Abcam), rabbit anti-FLAG (1:5,000; Sigma), rabbit anti-c-Myc (1:1,000; Santa Cruz Biotechnology), chicken anti-BAT3 (1:500; Abcam), mouse anti-FLAG M2 (1:2,000; Sigma), rabbit anti-ARP2 (1:1,000; Santa Cruz), mouse anti-TUBA4A (1:1,000; Santa Cruz), mouse anti-CyaA (1:5,000; Santa Cruz), rabbit anti-VCP (1:1,000; Abcam), and rabbit anti-GFP (1:1,000; Torrey Pines). The Western blots were revealed using ECL Plus Western blotting detection reagents (GE Healthcare Life Sciences).

Mass spectrometry and protein identification. In order to enrich for ubiquitinated species associated with FLAG-tagged LegU1, we performed a two-step immunoprecipitation. pMT123 (HA::Ub) and pCMV/3XFLAG::*legU1* were transfected into HEK-293T cells. After harvest, the lysates were first incubated with anti-FLAG M2 resin overnight at 4°C with gentle rotation to isolate LegU1-associated proteins. The resin was then loaded onto columns and subsequently washed three times with 10 ml lysis buffer and three times with 10 ml TBS. Immunoprecipitates were released by incubation of the resin with 150 ng/μl 3XFLAG peptide (Sigma). The volumes of the immunoprecipitates were adjusted in new lysis buffer, and the mixture was incubated with anti-HA resin overnight. After the resin was washed with lysis buffer and TBS, it was boiled in SDS sample buffer (without reducing agents) to release immunoprecipitates. Samples were loaded onto a precast SDS-polyacrylamide gel (Bio-Rad) and fractionated until the dye front migrated 2 to 3 cm into the gel. Control immunoprecipitations using the 3XFLAG vector and pMT123 were also performed. The gel was stained using SimplyBlue SafeStain (Invitrogen); and the regions of the gel that showed differential staining patterns between the 3XFLAG::*legU1* and 3XFLAG samples spanned from approximately >250 kDa, 150 to 250 kDa, and 60 to 150 kDa. For each sample, the regions of the gel corresponding to these sizes were cut out and separately analyzed by mass spectrometry to identify the proteins contained in each.

***In vitro* ubiquitination assays.** *In vitro* ubiquitination reactions were performed as described previously (17, 29, 43), with minor modifications. Briefly, HEK-293T cells were transfected with plasmids encoding FLAG::F-box (or the vector control) and untagged SKP1, CUL1, and RBX1. Eighteen to 24 h after transfection, lysates were harvested and processed as described above for the immunoprecipitation protocol with anti-FLAG M2 resin (Sigma). The washed resin was split into individual tubes (15 μl resin per reaction mixture) and incubated with a reaction mixture consisting of 1 μg biotin-*N*-ubiquitin (Boston Biochem) (this was excluded when detection was done with anti-BAT3 or anti-HA), 2.5 μg ubiquitin (Boston Biochem), 25 ng UBA1 (E1; Boston Biochem), 250 ng E2 (Boston Biochem), 1× energy-regenerating solution (Boston Biochem), and 0.5 μM ubiquitin aldehyde (Boston Biochem) with 4 mM magnesium acetate, 1 mM dithiothreitol (DTT), 100 μM phenylmethylsulfonyl fluoride; and the volume was adjusted to 10 μl using 20 mM HEPES, pH 7.6, 100 μM potassium acetate, and 1 mM DTT. The reactions proceeded for 90 min at 30°C and were stopped by boiling in SDS sample buffer for 5 min. These samples were separated by SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and detected using immunoblotting with either specific antibodies (as described above) or streptavidin-horseradish peroxidase (HRP; Invitrogen) to detect biotin N-ubiquitinated species.

RESULTS

Several *Legionella pneumophila* genes contain putative F-box domains and are translocated into the host cytosol during infection in a Dot/Icm-dependent manner. All strains of *L. pneumophila* sequenced have several genes with F-box motifs, with the Philadelphia-1 strain having at least five such genes (2, 16, 21) (Fig. 1B). In eukaryotic cells, endogenous F-box proteins largely function to target specific proteins for ubiquitination through the formation of a multiprotein complex, the SCF (Skp–Cullin–F-box) E3 ligase. As part of modular SCF complexes, each F-box protein generally contains two separate domains: one domain (the F-box motif) is required for interaction with the complex through Skp1, and other parts of the protein provide target specificity for the E3 ligase (Fig. 1A).

To determine if the F-box proteins are translocated via the Dot/Icm type IV secretion system, the *Bordetella pertussis* adenylate cyclase (CyaA) fusion assay was employed (63). This assay has successfully been used to identify translocated substrates (12, 18, 21, 32, 41, 62). The adenylate cyclase activity of CyaA depends on host calmodulin, so bacterial proteins fused to the enzyme do not produce increased intracellular cAMP levels unless they are first delivered to the host cytosol. The complete open reading frame of each of the five F-box proteins was fused to the 3′ end of the catalytic domain of *cyaA*, to allow the presence of any translocation signals to be revealed. The resulting plasmids were introduced into strains having an intact Dot/Icm system or a *dotA* mutation. Human macrophage-like U937 cells were challenged with each of these strains for 1 h, and intracellular cAMP levels were determined (Fig. 1C). Challenge of U937 cells with strains harboring each of the F-box fusion plasmids resulted in robust cAMP production compared to that by the cells challenged with a strain carrying the CyaA vector alone. This indicates that each F-box protein contains a translocation signal. This is consistent with previous studies that observe the presence of translocation signals in *legU1*, *legAU13*, and *ppgA* (20, 50). In each case, challenge with translocation-deficient (*dotA*) strains carrying each plasmid did not result in the production of intracellular cAMP levels above the CyaA vector background level (Fig. 1C). Taken together, these assays indicate that the Philadelphia-1 F-box proteins are substrates of the Dot/Icm translocation system, delivered to the host cytosol during infection.

It has been reported that many translocated substrates are upregulated during the post-exponential phase of *Legionella* growth (11), presumably to allow them to manipulate host cell functions at the earliest stages of intracellular growth. In order to determine the extent to which each of the F-box proteins is expressed, we used quantitative real-time RT-PCR to measure the transcript levels of each protein during culture of *L. pneumophila* in rich broth. The levels of expression of most of the *Legionella* F-box genes were relatively low compared to that for the translocated substrate SidC (Fig. 2). Furthermore, the transcription of these genes was not upregulated in post-expo-

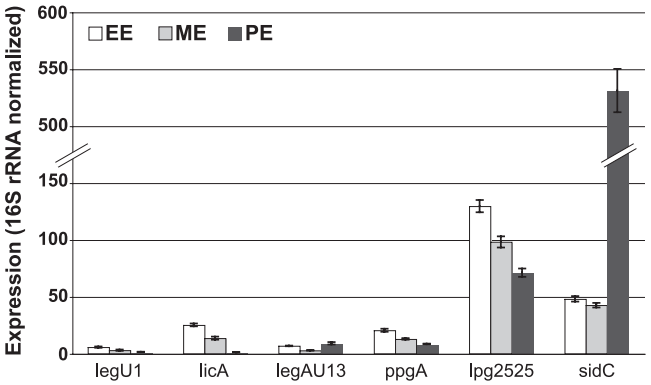

FIG. 2. Regulation of *L. pneumophila* F-box transcription in different phases of growth. The majority of the F-box proteins are transcribed at a low level and are not upregulated during post-exponential growth. Wild-type *L. pneumophila* was grown in broth until it reached early exponential phase (EE; A_{600} = 0.9), mid-exponential phase (ME; A_{600} = 2.0), or post-exponential phase (PE; A_{600} = 3.7) growth. Total RNA was purified from each and reverse transcribed, and individual levels of each transcript were measured using quantitative real-time RT-PCR. The levels of each transcript were normalized to the level of an endogenous control locus, 16S rRNA, the transcript level of which was set equal to 100,000 on this scale. For comparison, the transcript level of the translocated substrate, SidC, was also measured in each of these samples.

nential phase, in contrast to what was observed with the translocated substrate SidC (Fig. 2).

Two *Legionella* F-box proteins form a complex with SKP1 and CUL1 that associates with ubiquitinated species. To determine whether the predicted F-box motifs of each *Legionella* translocated substrate serve as functional F-box domains, we first determined if each protein could be found in a complex with human SKP1 in cultured cells transfected with plasmids encoding the translocated substrates. Previously, immunoprecipitation experiments have shown that one of the F-box proteins, LegAU13, can associate with cotransfected, tagged SKP1 (53) or endogenous SKP1 (40). This analysis has not been extended to other *L. pneumophila* F-box proteins, nor has it been demonstrated that this association leads to the formation of a functional SCF complex. To determine which of the five *L. pneumophila* Philadelphia-1 F-box genes associate with endogenous SKP1, each gene was cloned into a mammalian expression vector containing a 3XFLAG N-terminal epitope and introduced into human HEK-293T cells by transient transfection. Immunoprecipitation of the F-box proteins with anti-FLAG revealed that endogenous SKP1 was enriched in the LegU1, LicA, and LegAU13 immunoprecipitates compared to the levels in a vector-only control (Fig. 3A, anti-SKP1 panel, lanes IP). In contrast, SKP1 was not enriched in the PpgA or Lpg2525 immunoprecipitates. None of the immunoprecipitates were enriched for ARP2, an abundant endogenous protein used as a negative control (Fig. 3A, anti-ARP2 panel). LicA also associated strongly with an anti-SKP1-reactive band of unknown origin that migrated as a species that was slightly smaller than SKP1. These results are consistent with the presence of functional F-box domains within LegU1, LicA, and LegAU13, as each protein is capable of forming a complex with SKP1 in mammalian cells. In contrast, the F-box motifs previously predicted for PpgA and Lpg2525 may not represent functional F-box domains capable of facilitating SKP1 binding.

In order to determine if each of the F-box proteins was found in complexes with other host components of the SCF complex, we probed the anti-FLAG immunoprecipitates with antibody directed to CUL1, a critical scaffold protein of SCF complexes. Notably, endogenous CUL1 was enriched in FLAG::LegU1 and FLAG::LegAU13 immunoprecipitates (Fig. 3A, anti-CUL1 panel). CUL1 was not enriched in immunoprecipitates prepared from cells transfected with FLAG vector alone or any of the other F-box proteins, including LicA (Fig. 3A). Thus, LegU1 and LegAU13 associated with complexes containing both SKP1 and CUL1, while LicA coprecipitated with SKP1 but not with CUL1. The association of LicA with SKP1 in the absence of CUL1 suggests that LicA does not form an E3 ligase. Alternatively, LicA may form a noncanonical SCF complex using alternate eukaryotic components.

These anti-FLAG immunoprecipitates were next probed with anti-FLAG antibody, to confirm the immunoprecipitation of each 3XFLAG-tagged F-box protein (Fig. 3A, anti-FLAG panel). In each immunoprecipitate, this immunoblot analysis revealed an anti-FLAG reactive species of the predicted size for each tagged *L. pneumophila* protein (Fig. 3A, anti-FLAG panel, lanes IP, asterisks). This analysis also revealed the enrichment of multiple higher-molecular-weight FLAG-tagged species in the LegU1 and LegAU13 immunoprecipitates (Fig. 3A, anti-FLAG panel, lanes IP). The observation of such species under denaturing conditions is often indicative of post-translational modifications, including polyubiquitination. Ubiquitination of LegU1 and LegAU13 might be expected if each formed a functional E3 ligase complex, as F-box E3 ligases are known to autoubiquitinate, perhaps in order to facilitate turnover of the complex (38). The apparent lack of similar high-molecular-weight bands in the LicA immunoprecipitate argues against a role for LicA as an E3 ligase. LicA's association with SKP1 may modulate this host component and/or ubiquitination in a novel fashion, although LicA overexpression does not interfere with the association between LegU1 and SKP1 during transient transfection experiments (data not shown), arguing against an antagonistic role for LicA in the formation of SCF^{LegU1}.

In order to determine if the LegU1 and LegAU13-associated higher-molecular-weight species were ubiquitinated proteins, we transfected HEK-293T cells with plasmids encoding 3XFLAG-tagged LegU1 or LegAU13 and HA-tagged ubiquitin. Anti-FLAG immunoprecipitates were subjected to Western blot analysis, using anti-HA antibody (Fig. 3B, left panel) and anti-FLAG antibody (Fig. 3B, right panel). The overlap of ubiquitinated and F-box species in this experiment strongly suggests that LegU1 and (to a lesser extent) LegAU13 are ubiquitinated in human cells. The high-molecular-weight ubiquitinated species observed in these immunoprecipitates that do not react with the anti-FLAG antibody may represent ubiquitinated F-box protein with either obscured or deleted 3XFLAG epitopes, or they may represent endogenous ubiquitinated proteins associated with each complex.

Formation of heterologous SCF complexes with LegU1 and LegAU13 depends on an intact F-box domain. In order to determine if the SCF complexes formed with LegU1 and LegAU13 required the F-box domain of each F-box protein,

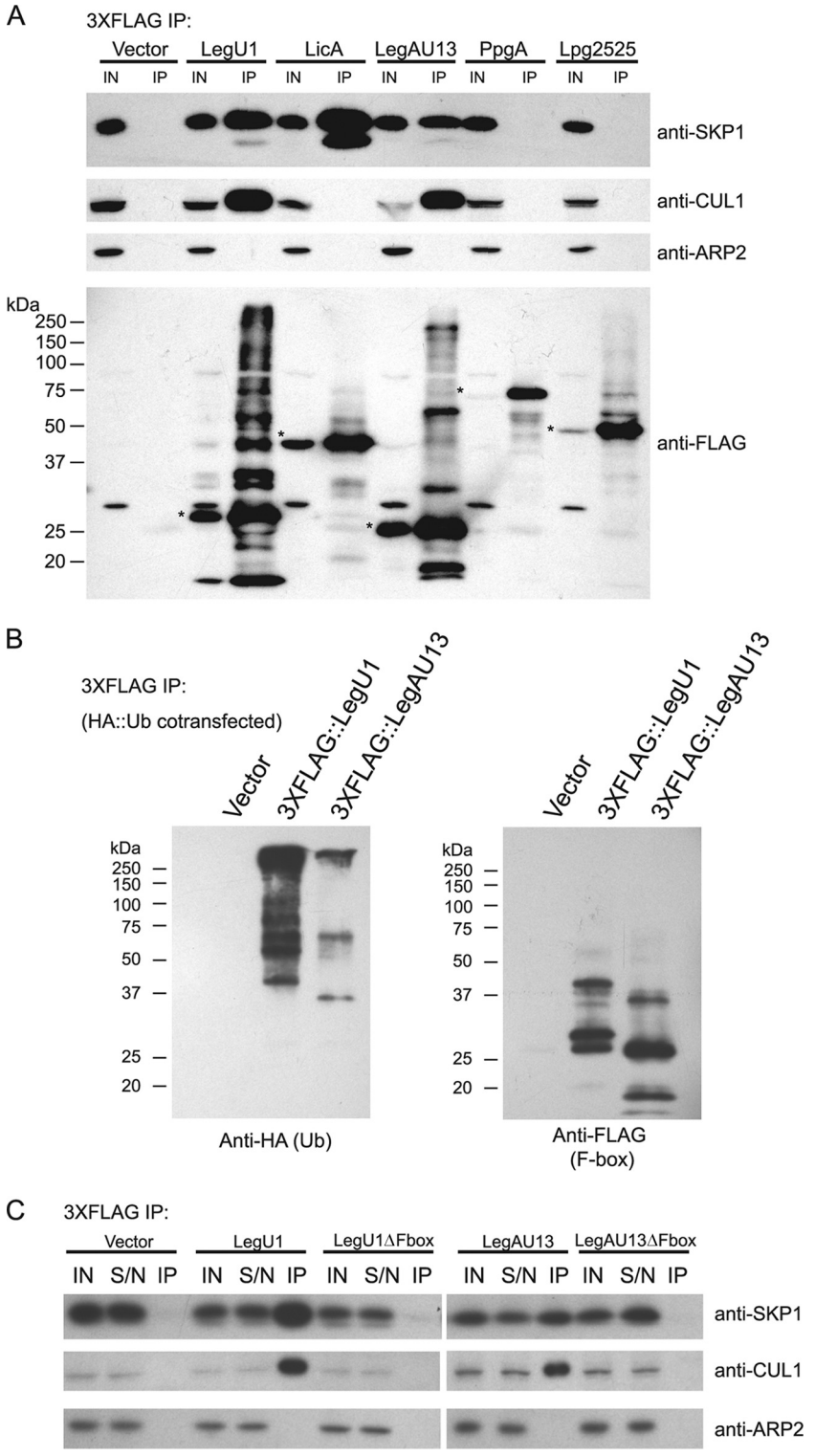

FIG. 3. A subset of *L. pneumophila* F-box proteins associate with SCF components in human cells. (A) Mammalian SCF components associate with a subset of *L. pneumophila* F-box proteins. HEK-293T cells were transfected with 3XFLAG-tagged *L. pneumophila* F-box proteins, and lysates were subjected to immunoprecipitation with anti-FLAG resin. The immunoprecipitates were analyzed by Western blot analysis using antibodies to endogenous human SKP1, CUL1, and ARP2, a negative control for nonspecific enrichment. Lanes IN, inputs, normalized to 1% of the amount of the immunoprecipitate (IP) fraction loaded on the gel (asterisks, predicted size of each 3XFLAG-tagged F-box protein). (B) Plasmids encoding 3XFLAG-tagged LegU1 and LegAU13 were cotransfected with a plasmid encoding HA-tagged ubiquitin in HEK-293T cells and subjected to anti-FLAG immunoprecipitation. Western blot analysis was performed using anti-HA antibody in order to reveal ubiquitinated species. After the membrane was stripped, it was probed with antibody directed against the FLAG epitope. (C) The association between SCF components and LegU1 and LegAU13 depends on their F-box domains. HEK-293T cells were transfected with plasmids encoding 3XFLAG-tagged LegU1 and LegAU13 with and without intact F-box domains. Cell lysates from these transfections were immunoprecipitated with anti-FLAG resin and analyzed by Western blotting using the noted antibodies (see Materials and Methods). Lanes S/N, unbound supernatant. Inputs and unbound supernatants were normalized to 1% of the immunoprecipitate fraction (lanes IP) loaded on the gel.

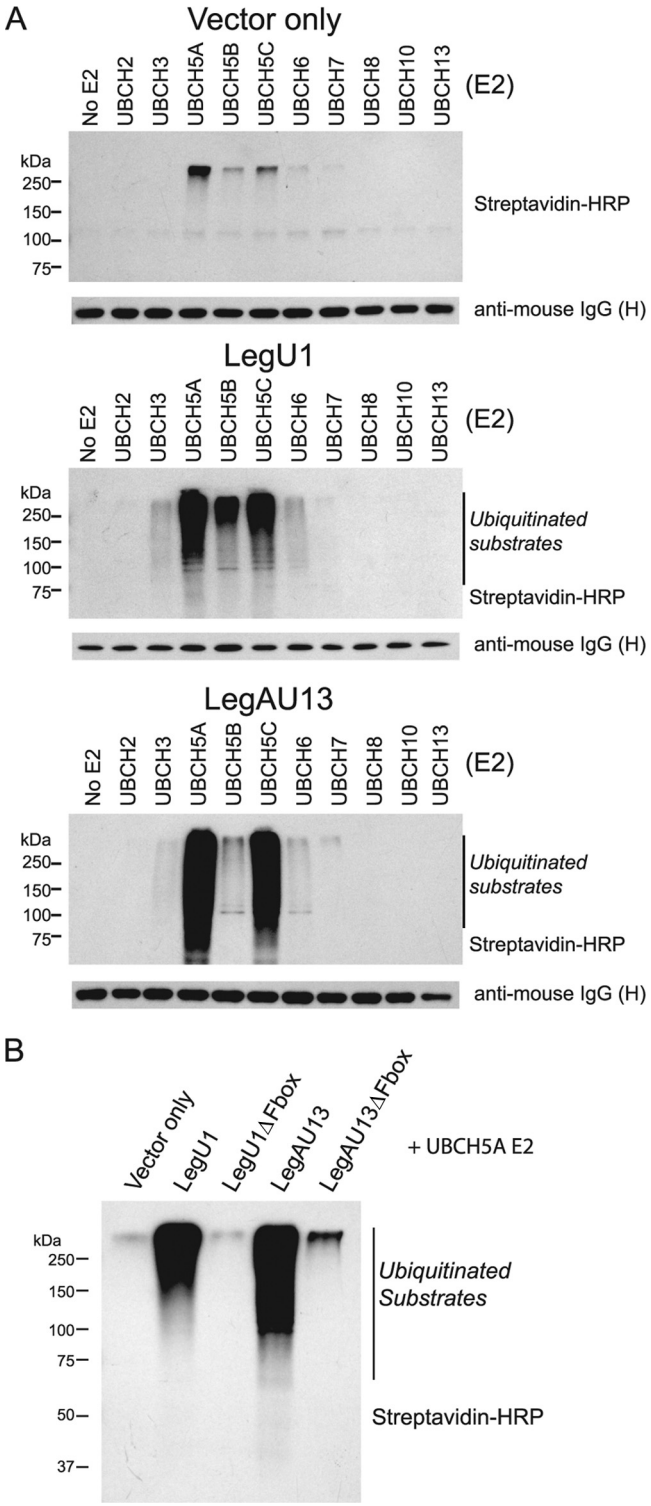

FIG. 4. LegU1 and LegAU13 associate with E3 ubiquitin ligase activity, as measured through an *in vitro* ubiquitination assay. (A) The E2 enzymes UBCH5A and UBCH5C support the robust generation of high-molecular-weight ubiquitinated species by LegU1 and LegAU13 *in vitro*. Ubiquitination reactions were performed *in vitro* (see Materials and Methods) using a panel of E2 enzymes, as noted above each displayed blot. Reactions were analyzed by Western blot analysis using HRP-conjugated streptavidin to detect biotin-ubiquitinated species and comparison of the results to those for a vector-only negative control. Western blot analysis was also performed using anti-mouse IgG antibody to identify any differences in the amount of SCF-bound resin aliquoted into each reaction mixture. (B) The F-box domains of LegU1 and LegAU13 are required for *in vitro* ubiquitination. *In vitro* ubiquitination reactions were performed in complete reaction mixtures that included the UBCH5A E2 enzyme, using either 3XFLAG-tagged LegU1, LegAU13, LegU1ΔF-box,or LegAU13ΔF-box or a vector-only control.

immunoprecipitation experiments were performed using LegU1 and LegAU13 constructs lacking functional F-box domains. HEK-293T cells were transfected with either wild-type or F-box-deleted FLAG::*legU1* and FLAG::*legAU13* plasmids. Lysates were subjected to immunoprecipitation with anti-FLAG resin, and immunoprecipitates were probed by immunoblot analysis using antibodies to SKP1, CUL1, and a negative control, ARP2. FLAG-tagged LegU1 and LegAU13 immunoprecipitates were enriched for SKP1 and CUL1 (but not for ARP2) (Fig. 3C). In contrast, when the F-box-domain sequences were deleted from LegU1 and LegAU13, these 3XFLAG-tagged mutant proteins did not immunoprecipitate endogenously expressed SKP1 or CUL1. These data indicate that the association between each F-box protein and SCF depends on the F-box domain. LicA protein lacking an intact F-box domain also does not immunoprecipitate SKP1 (data not shown). The loss of SKP1 and CUL1 enrichment in the immunoprecipitates from the F-box mutants is not due to degradation of the proteins, as each mutant shows similar steady-state levels and immunoprecipitation (data not shown).

SCF^{LegU1} and $SCF^{LegAU13}$ complexes associate with E3 ubiquitin ligase activity. On the basis of their interaction with multiple members of the host SCF E3 ligase, we next determined whether LegU1 and LegAU13 could support ubiquitination *in vitro*. To test this hypothesis, we employed a well-defined *in vitro* ubiquitination assay that has previously been used to study a number of F-box proteins (17, 29, 43). In this assay, the generation of high-molecular-weight biotin-ubiquitinated species is a readout for *in vitro* ubiquitination. Biotin-ubiquitinated species are likely a consequence of the autoubiquitination of SCF components (38) but could also result from the *in vitro* ubiquitination of F-box targets that coimmunoprecipitate with the complex. Briefly, a plasmid encoding 3XFLAG epitope-tagged F-box protein was transiently transfected into HEK-293T cells, along with plasmids encoding untagged SKP1, CUL1, and RBX1. Lysates of the transfected cells were prepared and anti-FLAG resin was used to immunoprecipitate SCF complexes. The washed resin binding to F-box protein-associated complexes was then mixed with the E1 ubiquitin-activating enzyme (Uba1), various E2 ubiquitin-conjugating enzymes, ATP, and biotinylated ubiquitin.

E3 ligases can rely on any of a number of E2-conjugating enzymes for their activity, so we incubated each SCF-bound resin with a panel of 10 human E2 enzymes (UBCH proteins) or with no E2 enzyme as a control. Compared to the findings for the vector-only control, which showed some basal ubiquitination (Fig. 4A, lanes UBCH5A, UBCH5B, and UBCH5C), both LegU1- and LegAU13-bound resins showed evidence of robust E3 ligase activity. For both F-box proteins, the E2 enzymes UBCH5A and UBCH5C (and, to a lesser extent,

UBCH5B) stimulated large amounts of ubiquitination, as determined by streptavidin-HRP detection of biotin-ubiquitinated species (Fig. 4A). Western blot analysis was also performed using anti-mouse IgG antibody to identify any differences in the amount of SCF-bound resin aliquoted into each reaction mixture (Fig. 4A, anti-mouse IgG). In order to determine if the F-box domain of each protein was required for this E3 ligase activity, we repeated these experiments using constructs lacking the F-box domains of LegU1 and LegAU13. In the presence of the E2 enzyme UBCH5A, the E3 ubiquitin ligase activity associated with LegU1 and LegAU13 was lost when the F-box domain of each was deleted (Fig. 4B), indicating that the F-box domain is necessary for this activity.

The host protein, BAT3, is a target of SCF^{LegU1} ubiquitination. In order to identify host targets of SCF^{LegU1} E3 ligase activity, we used a two-step enrichment strategy in which immunoprecipitates of 3XFLAG-tagged *Legionella* protein were subjected to a second round of immunoprecipitation to isolate ubiquitinated species. Due to its robust association with ubiquitinated species in transient transfection (Fig. 3B), we focused on LegU1 for these analyses. Plasmids encoding 3XFLAG-tagged LegU1 or the 3XFLAG-only vector control, together with HA-tagged ubiquitin, were transiently transfected in HEK-293T cells. After cell lysis, LegU1-associated proteins were isolated by anti-FLAG immunoprecipitation and were released from the resin using 3XFLAG peptide (see Materials and Methods). These eluates were then subjected to a second immunoprecipitation step using anti-HA resin in order to enrich for ubiquitinated substrates. After elution from the second resin, immunoprecipitates were fractionated on SDS-polyacrylamide gels and the bulk protein content of each was revealed by Coomassie staining. Unique species of high molecular weight appeared to be enriched in the 3XFLAG-tagged LegU1 immunoprecipitate relative to that in the 3XFLAG-only control (data not shown). For this reason, mass spectrometry was used to identify protein species within each of three fractions of high molecular weight (see Materials and Methods). Compared to the 3XFLAG-only vector control, unique LegU1-associated peptides included peptides from SKP1 and CUL1, as predicted, as well as several peptides corresponding to HLA-B-associated transcript 3 (BAT3) (data not shown). BAT3 is a highly expressed protein with a broad range of roles in the mammalian cell and has been implicated in modulating apoptosis, the response to ER stress, p53-regulated expression, and Hsp70 stability (23, 24, 57, 66).

Both to confirm the association between LegU1 and BAT3 and to determine if it was specific to LegU1, we performed anti-BAT3 immunoblot analysis with the anti-FLAG immunoprecipitates from each of the five 3XFLAG-tagged *Legionella* F-box proteins (detailed earlier for Fig. 3A). The levels of BAT3 in each input lane (normalized for total protein) did not change (Fig. 5A, anti-BAT3 panel, lanes IN). Immunoblot analysis revealed that endogenous BAT3 was enriched in the 3XFLAG-tagged LegU1 immunoprecipitate compared to the level in a vector-only control (Fig. 5A, anti-BAT3 panel, lanes IP). In contrast, BAT3 was not enriched in the anti-FLAG immunoprecipitates from 3XFLAG-tagged LegAU13, LicA, or PpgA lysates. A very small amount of BAT3 was present in the Lpg2525-containing immunoprecipitate. A control for nonspecific enrichment, VCP/p97/CDC48, was not enriched in any of the immunoprecipitates. Taken together, these data indicate that LegU1 associates with BAT3.

We determined if the association observed between LegU1 and BAT3 depended on the F-box domain of LegU1, as sequences outside the F-box domain are generally thought to provide substrate specificity to the SCF complex. HEK-293T cells were transfected with plasmids encoding 3XFLAG-tagged LegU1, 3XFLAG-tagged LegU1 with a deleted F-box domain, or 3XFLAG-only vector as a control. Lysates from these cells were subjected to anti-FLAG immunoprecipitation and were analyzed by immunoblot analysis with anti-BAT antibodies. BAT3 was enriched in both the LegU1 and LegU1ΔFbox immunoprecipitates compared to the levels in the 3XFLAG-only control (Fig. 5B). None of the immunoprecipitates were enriched for tubulin alpha-4A (TUBA4A), an abundant endogenous protein used as a negative control (Fig. 5B, anti-TUBA4A panel). This indicates that the association between LegU1 and BAT3 does not depend on the F-box domain. This association is also presumably independent of SKP1 or CUL1, as an SKP1-CUL1-LegU1 complex was previously shown to not form in the absence of this domain (Fig. 3A).

The association between BAT3 and LegU1 was further analyzed by reciprocal coimmunoprecipitation. HEK-293T cells were transfected with combinations of plasmids encoding a Myc-only vector, Myc-tagged BAT3, the 3XFLAG-only vector, and 3XFLAG-tagged LegU1. Lysates from these cells were subjected to anti-Myc immunoprecipitation and were analyzed by anti-FLAG immunoblotting. Immunoprecipitates from lysates containing both Myc-tagged BAT3 and 3XFLAG-tagged LegU1 showed enrichment for LegU1 compared to the level in a Myc-only immunoprecipitate (Fig. 5C). 3XFLAG-tagged LegU1 migrating at the predicted size (Fig. 5C, asterisk) is enriched in the Myc-tagged BAT3 immunoprecipitate, along with anti-FLAG-reactive species of higher molecular weight. These data are consistent with the association of BAT3 with both the unmodified and modified (ubiquitinated) forms of LegU1. The reduced level of 3XFLAG-tagged LegU1 in the Myc-only input lane (Fig. 5C, compared to that in the Myc-tagged BAT3 input lane) was also observed when other unrelated 3XFLAG constructs were cotransfected with the Myc-only vector (data not shown).

To determine if BAT3 can serve as a substrate of LegU1-mediated polyubiquitination, *in vitro* ubiquitination experiments were performed. As before, a plasmid encoding 3XFLAG-tagged LegU1 was transiently transfected into HEK-293T cells, along with plasmids encoding untagged SKP1, CUL1, and RBX1. 3XFLAG-tagged LegU1 was immunoprecipitated using anti-FLAG resin and introduced into a complete ubiquitination reaction mixture, including the E1 ubiquitin-activating enzyme, the UBCH5A E2 protein, and wild-type ubiquitin. Immunoblot analysis with anti-BAT3 antibody was used to probe this *in vitro* reaction for endogenous BAT3. BAT3 was enriched in the *in vitro* reactions using LegU1 immunoprecipitates relative to the 3XFLAG-only vector control (Fig. 5D). This immunoblot analysis also revealed that higher-molecular-weight species of BAT3 were enriched in the LegU1 immunoprecipitate incubated with wild-type (WT) ubiquitin (Ub) and the E1 enzyme [Fig. 5D, LegU1, lanes +E1 and +Ub(WT)].

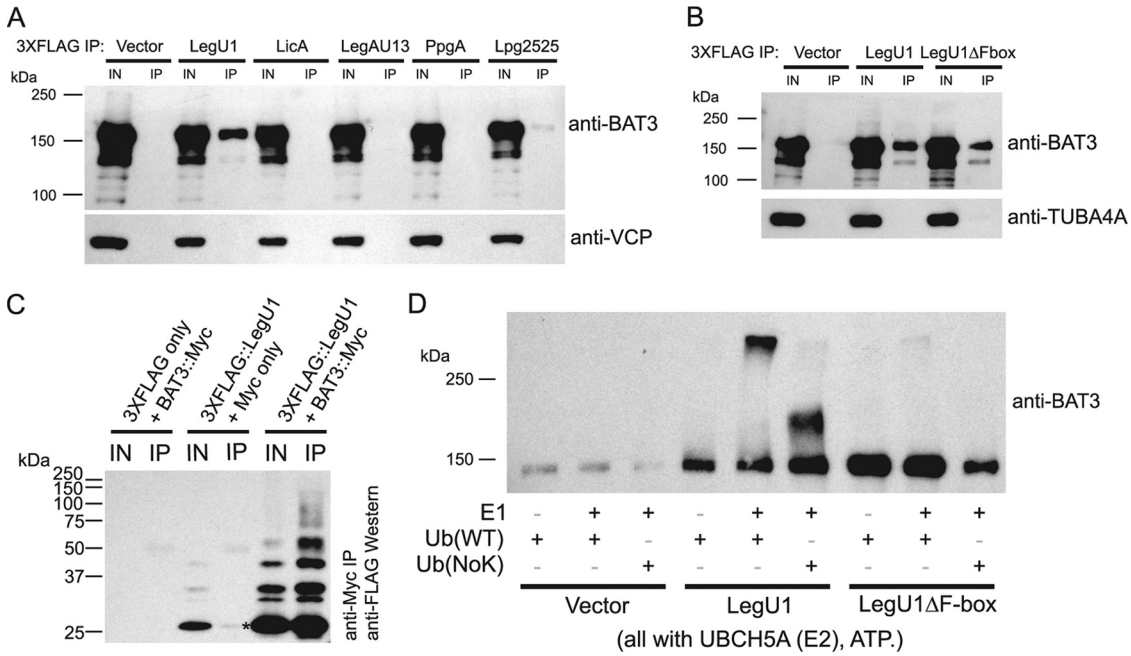

FIG. 5. LegU1 associates with the host protein BAT3 and can direct its ubiquitination. (A) Among the five Philadelphia-1 F-box proteins, only LegU1 associates with BAT3. HEK-293T cells were transfected with plasmids encoding 3XFLAG-tagged LegU1, LicA, LegAU13, PpgA, or Lpg2525. Cell lysates from these transfections were immunoprecipitated with anti-FLAG resin and analyzed by Western blotting using anti-BAT3 antibody or anti-VCP as a control for nonspecific enrichment. Lanes IN, inputs; lanes IP, immunoprecipitates. (B) LegU1 associates with BAT3 independently of the F-box domain. HEK-293T cells were transfected with 3XFLAG-tagged LegU1, LegU1ΔF-box, or the 3XFLAG vector alone. After anti-FLAG immunoprecipitation, samples were subjected to Western blot analysis using anti-BAT3 antibody and anti-TUBA4A antibody as controls for nonspecific enrichment. (C) Immunoprecipitation of LegU1 by BAT3. HEK-293T cells were transfected with either Myc-tagged BAT3 and the 3XFLAG vector, 3XFLAG-tagged LegU1 and the Myc vector, or Myc-tagged BAT3 together with 3XFLAG-tagged LegU1. Lysates from these cells were subjected to anti-Myc immunoprecipitation, and immunoblot analysis was performed using anti-FLAG antibody. Transfection combinations are noted above the displayed blot (asterisk, predicted size of 3XFLAG-tagged LegU1). For panels A to C, each input fraction was normalized to 1% of the immunoprecipitate fraction loaded on the gel. (D) LegU1 directs the polyubiquitination of BAT3 *in vitro* in a manner dependent on the F-box domain. Resin harboring 3XFLAG-tagged LegU1 or LegU1ΔF-box was prepared by anti-FLAG immunoprecipitation from HEK-293T cells transfected with each translocated substrate. Aliquots of this resin were used for *in vitro* ubiquitination experiments (see Materials and Methods) in a complete reaction mixture with wild-type ubiquitin [Ub(WT)], E1 enzyme, and an E2 enzyme, UBCH5A. Using other aliquots of the same resin, parallel reactions were performed using a reaction mixture lacking the E1 enzyme or containing only an isoform of ubiquitin (NoK) that lacks all lysine residues and that is incapable of extending polyubiquitin chains. Western blot analysis using anti-BAT3 antibody was used to detect any endogenous BAT3 protein that coimmunoprecipitated with complexes and was subjected to these reactions.

To further characterize the shift in the apparent molecular weight of BAT3 during *in vitro* ubiquitination reactions, two parallel reactions with SCF^{LegU1} were performed. First, a reaction was performed without the E1 enzyme, a crucial component in ubiquitination reactions. High-molecular-weight BAT3 species did not accumulate in this control reaction, as revealed by anti-BAT3 immunoblot analysis [Fig. 5D, LegU1, lanes −E1 and +Ub(WT)]. This is consistent with the molecular weight shift being due to the ubiquitination of BAT3, as ubiquitination cannot occur without an E1 enzyme. Second, an *in vitro* ubiquitination reaction was performed using an ubiquitin mutant protein (NoK) in which arginine has been substituted for all seven lysines. Lysine residues within ubiquitin are required for polyubiquitination; thus, this Ub(NoK) mutant is incapable of forming polyubiquitin chains (3). Anti-BAT3 immunoblot analysis of the Ub(NoK) reaction [Fig. 5D, lanes +E1, −Ub(WT), +Ub(NoK)] revealed a greatly diminished shift in the BAT3 molecular weight relative to that observed in the complete reaction mixture containing both E1 and wild-type ubiquitin [Fig. 5D, lanes +E1 and +Ub(WT)], consistent with single or multiple monoubiquitination of BAT3. This modification of BAT3 also required the F-box domain of LegU1, as similar shifts were not observed in reactions using LegU1ΔF-box (Fig. 5D, lanes LegU1ΔF-box). Taken together, the observed shifts in the molecular weight of BAT3 in the complete ubiquitination reaction mixture are consistent with the depletion of nonubiquitinated BAT3 and the formation of a polyubiquitinated higher-molecular-weight form.

In mammalian cells, LegU1 also associates with another translocated substrate, Lpg2160. In addition to the five F-box proteins, a large repertoire of other *Legionella* proteins is delivered to the host cytosol during infection. Therefore, we hypothesized that LegU1 and/or the LegU1-BAT3 complex might interact with one or more of these other translocated substrates. To test this, 3XFLAG-tagged LegU1ΔF-box was transiently expressed in HEK-293T cells, along with complex pools containing 23 GFP-tagged *Legionella* proteins from a library of over 150 putative substrates of the type IV secretion system (41). Lysates from each of these pools were subjected to anti-FLAG immunoprecipitation, separated on SDS-polyacrylamide gels, and probed by anti-GFP immunoblotting. One pool consistently showed coimmunoprecipitation of anti-GFP-

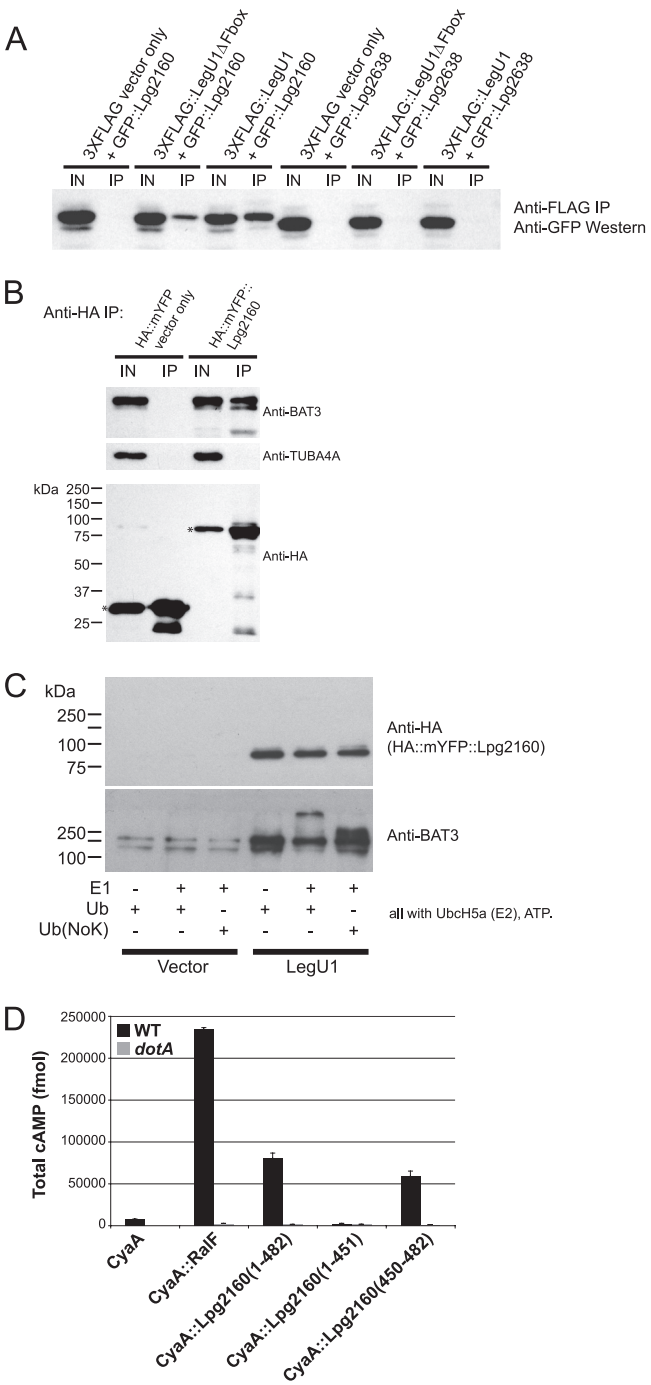

FIG. 6. A second *L. pneumophila* translocated substrate, Lpg2160, associates with BAT3 and LegU1 in mammalian cells. (A) The *L. pneumophila* protein Lpg2160, but not its close paralog, Lpg2638, associates with LegU1 independently of the F-box domain. HEK-293T cells were cotransfected with plasmids encoding the 3XFLAG vector, 3XFLAG::LegU1, or 3XFLAG::LegU1ΔF-box, along with GFP-tagged Lpg2160 or Lpg2638. Cell lysates were subjected to anti-FLAG immunoprecipitation, followed by anti-GFP immunoblot analysis. Lanes IN, inputs; lanes IP, immunoprecipitates. (B) Lpg2160 associates with BAT3 independently of LegU1. HEK-293T cells were transfected with either the HA::mYFP vector or HA::mYFP-tagged Lpg2160, and cell lysates were subjected to immunoprecipitation with anti-HA resin. Western blot analysis was performed using anti-BAT3. In addition, anti-HA antibodies were used to detect ectopically expressed HA::mYFP-tagged proteins (asterisk), and anti-TUBA4A was used as a control for nonspecific enrichment. For panels A and B, each input fraction was normalized to 1% of the immunoprecipitate fraction loaded on the gel. (C) Lpg2160 does not serve as a target of ubiquitination by LegU1 *in vitro*, despite its coassociation. *In vitro* ubiquitination reactions were performed as described in the text using resin containing 3XFLAG or 3XFLAG::LegU1 (see Materials and Methods). Aliquots of resin were incubated in either the complete reaction mixture, reaction mixtures lacking the E1 enzyme, or reaction mixtures using a mutant form of ubiquitin (NoK) incapable of forming polyubiquitin chains. After the reactions were stopped, samples were analyzed by Western blotting using anti-HA or anti-BAT3 antibodies. (D) Lpg2160 is translocated into the host cytosol in a Dot/Icm-dependent manner. U937 cells were challenged with wild-type or translocation-deficient *dotA* bacteria expressing CyaA (negative control) or CyaA fused to full-length Lpg2160(1-482), Lpg2160(1-451), or Lpg2160(450-482). After 1 h, host cells were lysed and cAMP levels were quantified. The data represented are the averages of three replicates ± standard errors of the means.

reactive species (data not shown). HEK-293T cells were transfected with 3XFLAG-tagged LegU1ΔF-box and the individual GFP-tagged effectors contained within this pool. Lysates from these transfections were subjected to anti-FLAG immunoprecipitation and probed by anti-GFP immunoblotting (data not shown). We found that the putative translocated substrate, Lpg2160, consistently immunoprecipitated with 3XFLAG::LegU1 and 3XFLAG::LegU1ΔF-box when it was coexpressed in mammalian cells (Fig. 6A). The closely related paralog of Lpg2160, Lpg2638, was not enriched in any of the immunoprecipitations (Fig. 6A, Lpg2638, lanes IP).

BAT3 associates with Lpg2160 in mammalian cells. Because LegU1 associates with BAT3 in mammalian cells, we next determined whether the LegU1-Lpg2160 interaction was due to the association of Lpg2160 with a BAT3-LegU1 complex formed in these cells. To determine if Lpg2160 binds to BAT3 in the absence of LegU1, we transfected HEK-293T cells with plasmids encoding HA::mYFP-tagged Lpg2160. Lysates from these cells were subjected to anti-HA immunoprecipitation, followed by blotting with anti-BAT3 (Fig. 6B, anti-BAT3). BAT3 was enriched in the Lpg2160 immunoprecipitate relative to the level for an HA::mYFP-only vector control (Fig. 6B, lanes IP). The negative control, TUBA4A, was not enriched in either immunoprecipitate (Fig. 6B, anti-TUBA4A panel). Anti-HA immunoblot analysis revealed enrichment for HA::mYFP and HA::mYFP::Lpg2160 in each corresponding immunoprecipitate (Fig. 6B, anti-HA, lanes IP), indicating that the failure to detect immunoprecipitation of BAT3 in the vector-only control was not due to the absence of bait.

The association of Lpg2160 with both BAT3 and LegU1 indicated that it could be similarly targeted for ubiquitination by the F-box protein. *In vitro* ubiquitination reactions were performed to determine if LegU1 could direct the ubiquitination of Lpg2160. HEK-293T cells were transfected with plasmids encoding 3XFLAG-tagged LegU1 and HA::mYFP-tagged Lpg2160, together with SKP1, CUL1, and RBX1. Lysates from these cells were subjected to anti-FLAG immunoprecipitation. Washed resin was incubated with the *in vitro* ubiquitination reaction mixture as described before. As described above, immunoblot analysis with anti-BAT3 an-

tibody indicated that BAT3 was ubiquitinated *in vitro*, suggesting that Lpg2160 does not block the reaction (Fig. 6C, anti-BAT3). In contrast, there was no evidence for species of increased molecular weight after the same blot was probed for HA::mYFP::Lpg2160 (Fig. 6C, anti-HA). The anti-HA-reactive species in each of the LegU1 reactions is also not depleted in the wild-type reaction compared to the level for each control, arguing that the failure to observe high-molecular-weight species was not due to the formation of polyubiquitinated species with inaccessible HA epitope tags. Taken together, these data indicate that while LegU1 can direct the polyubiquitination of BAT3 *in vitro*, it does not appear to ubiquitinate Lpg2160.

Lpg2160 is a Dot/Icm substrate translocated into the host cytosol during infection. To determine if Lpg2160 was a translocated substrate of the Dot/Icm system, a plasmid encoding the complete open reading frame of Lpg2160 fused to the 3′ end of *cyaA* was generated. This plasmid was introduced into strains with an intact Dot/Icm system or a *dotA* mutation. Human U937 macrophage-like cells were challenged with these bacteria, and after 1 h, intracellular cAMP levels were determined (Fig. 6D). Challenge of U937 cells with a wild-type strain harboring the *cyaA*::*lpg2160* fusion plasmid resulted in robust cAMP production relative to the level for cells challenged with a strain carrying the CyaA vector alone. This result is consistent with the presence of a translocation signal within the open reading frame of *lpg2160*. As a control, U937 cells were also challenged with strains expressing CyaA fused to RalF, a known translocated substrate (49). As expected, challenge with a CyaA::RalF-expressing strain also resulted in increased cAMP levels (Fig. 6D). To determine if the observed production of cAMP was Dot/Icm dependent, U937 cells were challenged with *dotA* mutant bacteria expressing the CyaA::Lpg2160 fusion protein. Challenge with this *dotA* strain did not result in increased levels of cAMP compared to the levels measured from cells challenged with bacteria harboring the CyaA vector alone. This indicates that Lpg2160 is a translocated substrate of the Dot/Icm system.

The C-terminal sequence of Lpg2160 was previously suggested to be a putative translocation signal of the Dot/Icm system, determined by a machine-learning approach (10). To determine if the translocation signal resides in this region of Lpg2160, plasmids that encode CyaA fusion proteins with either a 31-amino-acid C-terminal truncation of Lpg2160 [CyaA::Lpg2160(1-451)] or the last 32 amino acids of Lpg2160 [CyaA::Lpg2160(450-482)]. These plasmids were introduced into strains with either an intact Dot/Icm system or a *dotA* mutation. Increased cAMP levels were observed in U937 cells challenged with bacteria expressing CyaA::Lpg2160(450-482) but not in cells challenged with bacteria expressing CyaA::Lpg2160(1-451) (Fig. 6D). The increased level of cAMP observed after challenge with bacteria expressing CyaA::Lpg2160(450-482) was Dot/Icm dependent, as no increase in cAMP levels was observed upon challenge with the corresponding *dotA* strain (Fig. 6D). Western blot analysis with anti-CyaA antibody showed the robust expression of each fusion protein (data not shown), indicating that the differences in cAMP levels observed were not due to variations in protein stability or expression. These data suggest that there is a Dot/Icm-dependent translocation signal located within the last 32 amino acids of Lpg2160. This signal appears to be both necessary and sufficient for the efficient translocation of Lpg2160 into the host cytosol.

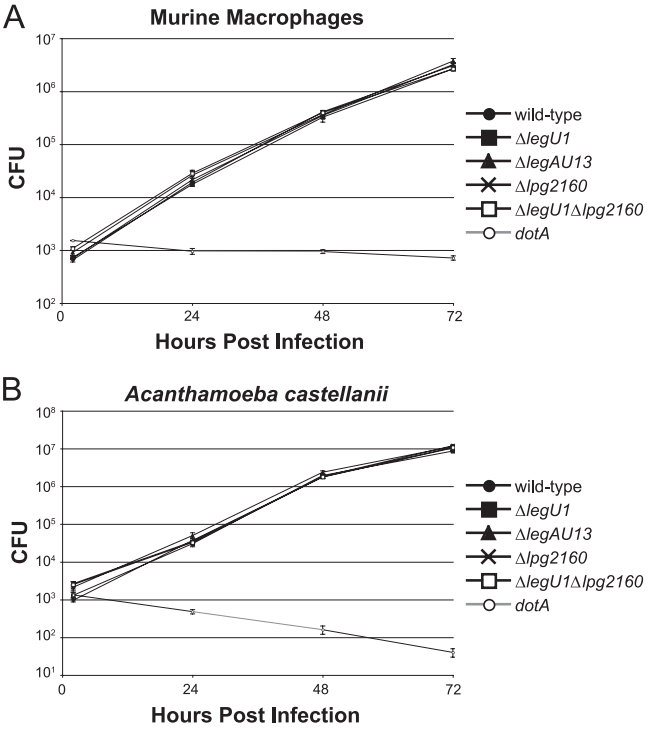

FIG. 7. LegU1, LegAU13, and Lpg2160 are dispensable for intracellular growth in both primary mouse macrophages and amoebae. Intracellular growth of *L. pneumophila* within A/J mouse primary bone marrow-derived macrophages (A) and the amoeba *Acanthamoeba castellanii* (B). Host cells were challenged with the indicated *L. pneumophila* strains: the wild type; Δ*legU1*, Δ*legAU13*, Δ*lpg2160*, Δ*legU1*, and Δ*lpg2160* strains; and a *dotA* mutant. The number of viable bacteria at each time point was determined by lysing host cells and plating dilutions of bacteria onto CYET plates. The data were plotted as the averages of three experiments per strain per time point. Error bars represent the standard errors of the means of these measurements.

Effects on intracellular growth. Disruption of one *Legionella* F-box gene, *legAU13*, has been reported to severely limit intracellular replication of the AA100 laboratory strain of *L. pneumophila* in a variety of hosts, including mammalian macrophages (1, 52, 53). To determine if, in the Philadelphia-1 strain, *legAU13*, *legU1*, or *lpg2160* mutants were deficient in intracellular growth, in-frame deletions of each were constructed. These strains, along with wild-type and a Dot/Icm-deficient *dotA* mutant, were grown to post-exponential phase in broth. Primary bone marrow-derived A/J mouse macrophages, which are permissive for *L. pneumophila* replication, were challenged with these strains, and the intracellular growth of each strain was measured by lysing macrophages every 24 h and plating dilutions of each lysate on bacteriological plates for CFU analysis (Fig. 7). We observed no discernible growth defect for the *legAU13*, *legU1*, or *lpg2160* mutants relative to the growth for the wild-type strain in either primary bone marrow-derived A/J mouse macrophages (Fig. 7A) or the amoeba *Acanthamoeba castellanii* (Fig. 7B). The *dotA* mutant does not replicate in either host cell type, as described previ-

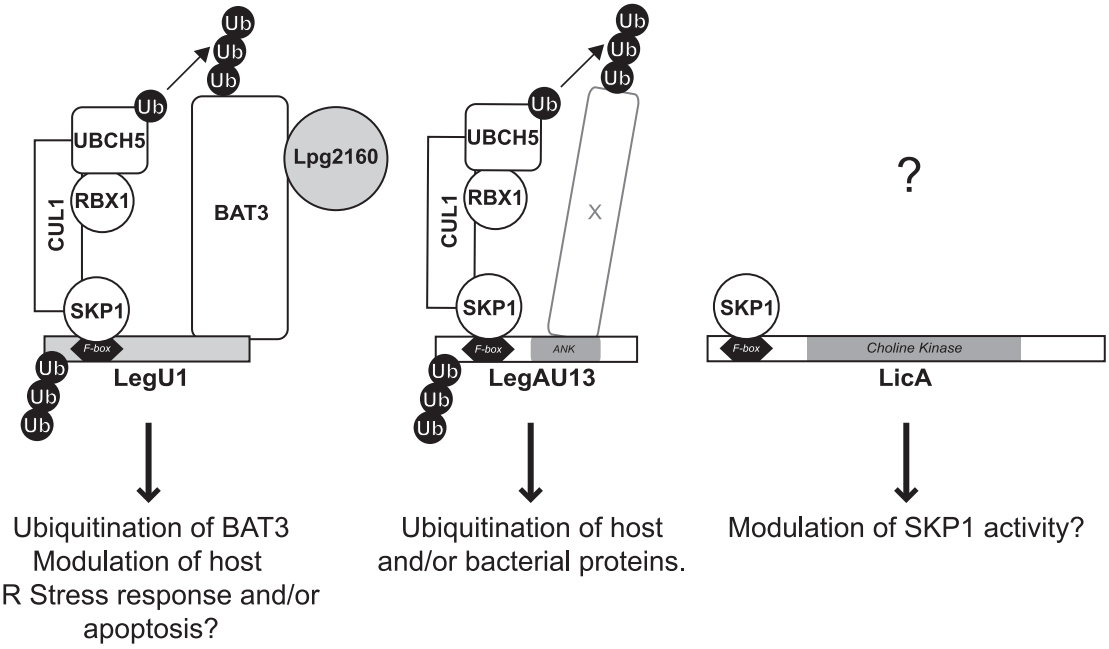

FIG. 8. Composition and potential role of each *L. pneumophila* F-box-associated complex in mammalian cells. On the basis of the results of our experiments, we propose a model in which LegU1 and LegAU13 form intact SCF complexes with E3 ligase activity. SCF^{LegU1} directs the ubiquitination of the host protein BAT3, which may result in a reduced ER stress response or suppression of host apoptotic pathways. Another *L. pneumophila* translocated substrate, Lpg2160, also associates with the LegU1-BAT3 complex but does not get ubiquitinated. $SCF^{LegAU13}$ is an E3 ubiquitin ligase complex with unknown target affinity. In addition to being subject to autoubiquitination, we hypothesize that LegAU13 directs the ubiquitination of host and/or bacterial proteins during intracellular replication. The F-box protein LicA associates with SKP1 but does not associate with CUL1. The consequences of this interaction are unknown, but we propose that LicA-SKP1 complexes may not direct the ubiquitination of target proteins.

ously (6, 9). Similar results were obtained using a single-cell infectious center assay (22) to measure intracellular replication in A/J mouse macrophages (data not shown). We hypothesized that LegU1 and Lpg2160 might serve overlapping functions during infection, because they both associate with BAT3. A double *legU1 lpg2160* mutant strain was also constructed; however, we observed no discernible growth defect for this double mutant. These results are consistent with previous observations that *L. pneumophila* strains harboring multiple deletions of translocation substrates often do not display gross defects in intracellular growth (42).

DISCUSSION

We have demonstrated that each of the known F-box motif proteins in *L. pneumophila* Philadelphia-1 is translocated to the host cytosol in a Dot/Icm-dependent manner. The established role of F-box domains is to facilitate an interaction with SKP1, a core component of the SCF E3 ligase complex. Prokaryotes do not encode SKP1, CUL1, or RBX1, so any E3 ligase activity promoted by the *L. pneumophila* F-box proteins almost certainly requires SCF components from the host cell, with the prokaryotic F-box providing substrate specificity for the entire complex. By analysis of each protein in mammalian cells, we have confirmed that three of the *L. pneumophila* F-box domain-containing proteins associate with endogenous SKP1 in mammalian cells (Fig. 8). Two of these proteins form canonical E3 ligases composed of the bacterial F-box protein and host components of the SCF. These hybrid SCF complexes can support polyubiquitination *in vitro*, suggesting that they form catalytically active E3 ligases, after assembly in the host cytosol, capable of directing the ubiquitination of target substrates. Another *L. pneumophila* F-box protein, LicA, can form a complex with SKP1 in mammalian cells that appears to exclude other members of the SCF complex. LicA may form a noncanonical SCF complex, or it may bind to SKP1 for reasons independent of SCF formation that interfere with formation of an SCF complex. Proteomic analysis of LicA and its interacting partners should provide further insight into this substrate's function.

We have identified two *L. pneumophila* translocated substrates that associate with the host protein BAT3, a key regulator of several important processes, including apoptosis and the response to ER stress (24, 66). The association of both LegU1 and Lpg2160 with BAT3 appears to be nonexclusive, and our data support a model in which the multicomponent SCF^{LegU1} E3 ligase, Lpg2160, and BAT3 form a complex. LegU1 E3 ligase activity can direct the ubiquitination of BAT3 but does not direct the ubiquitination of Lpg2160, despite the coassociation. This result suggests that this coassociation is not sufficient to result in targeting by the SCF^{LegU1} E3 ligase.

L. pneumophila co-opts host vesicle trafficking during infection (13, 20, 32, 33, 44, 45, 48, 49, 54, 62), and several Dot/Icm translocated substrates have been shown to protect host cells against sources of cytotoxic stress during infection (5, 39, 61). Ablation of BAT3 in mammalian cells has been shown to confer resistance to several inducers of ER stress (23, 24). The

association of BAT3 with at least two *L. pneumophila* translocated substrates raises the intriguing possibility that the pathogen co-opts or modulates BAT3 activity during infection in order to modulate host ER stress responses. We propose that, along with other effectors, LegU1 and Lpg2160 may function to mitigate the effects of disrupting normal vesicular trafficking in the host cell, perhaps by interfering with BAT3 activity. The findings from experiments directed at measuring the intracellular replication of the *legU1* mutant in macrophages under conditions of exogenous ER stress induction are thus far inconclusive (data not shown). This approach can, however, be extended to the study of other mutant strains, such as the *legU1 lpg2160* double mutant, and/or other conditions of host cell stress. BAT3 has also been shown to modulate other processes in the mammalian cell, including Hsp70 stability (19), as well as p53-regulated expression in response to DNA damage (57).

The *L. pneumophila* F-box protein LegAU13 forms a functional E3 ligase complex in mammalian cells. The role of $SCF^{LegAU13}$ is presumably to target host or bacterial factors for ubiquitination. Overexpressed LegAU13 localizes to the periphery of mammalian cells (52, 53), suggesting that the target of LegAU13 may also reside in or around the plasma membrane. The Paris strain ortholog of LegAU13, Lpp2082, has been shown to associate with the host protein PARVB; however, this association does not result in the polyubiquitination of PARVB (40). One possibility is that PARVB acts as an accessory protein to direct LegAU13 to the plasma membrane, where the E3 ligase activity of $SCF^{LegAU13}$ directs the ubiquitination of one or more additional proteins. The approach that we used to identify BAT3 as a target of LegU1 ubiquitination may also work for LegAU13, with one complication being that fewer ubiquitinated species associate with LegAU13 during immunoprecipitation experiments. One intriguing possibility is that LegAU13 may target one or more bacterial substrates for ubiquitination. In such a case, the reduced association between LegAU13 and ubiquitinated species (compared to the level of association for LegU1) might be a consequence of analyzing the LegAU13 activity through heterologous expression in mammalian cells and in isolation from other bacterial proteins.

The role of LegAU13 in intracellular replication and ubiquitination of LCV-associated proteins is partially obscured by the striking discrepancy between phenotypes observed by different groups (35, 53). This lack of concordance may be due to subtle differences in strain construction, but more likely, it may result from differences between the parental strains of *L. pneumophila* used in each laboratory. While no intracellular replication or ubiquitin accumulation defects have been observed for *legAU13* (*ankB*) mutant strains generated in the Philadelphia-1 strain background (Lp01, Lp02) (35), those generated in a different *L. pneumophila* strain, 130b/AA100, display severe defects in both intracellular growth and polyubiquitin accumulation (1, 52, 53). The Philadelphia-1 and AA100 strains of *L. pneumophila* are likely quite different in a number of respects, as the AA100 strain of *L. pneumophila* was isolated from the Wadsworth Veterans Administration Hospital in Los Angeles, CA (27). While some genetic differences between the strains have been described (56), whole-genome resequencing efforts using next-generation sequencing technologies should be of particular use in determining what, if any, genetic differences underlie these differences in observed phenotypes (46). The insight garnered from these analyses should facilitate the dissection of the role of LegAU13 during intracellular replication.

The modulation of the host ubiquitin system by *L. pneumophila* likely extends beyond the F-box proteins. For instance, the genome of the Philadelphia-1 strain (along with those of related strains Lens, Paris, and Corby) contains at least one protein with homology to ubiquitin-like proteases, Lpg2907, that has been identified to be a putative deubiquitinase (15). In addition to F-box proteins, another type of E3 ligase, the U-box containing LegU2/LubX has also been identified in *L. pneumophila* and has been described to target the host factor Clk1 for ubiquitination (21, 37). Additionally, it has previously been shown that polyubiquitinated proteins accumulate around the LCV during infection of host cells with wild-type bacteria but not Dot/Icm-deficient bacteria (25). This polyubiquitin signal likely represents ubiquitinated species from one or more of the following sources: the active ubiquitination of bacterial proteins at the LCV by host or bacterial E3 ligases, the ubiquitination of host proteins at the LCV by bacterial factors, or the trafficking of ubiquitinated species to the surface of the vacuole. The results of time course experiments strongly suggest that at least some of this ubiquitination may be directly tied to the proper translocation of Dot/Icm substrates into the cytosol or VCP/Cdc48-dependent removal of substrates from the LCV surface after translocation (25). Directed, proteomic analysis of these LCV-associated, ubiquitinated proteins will likely answer many of these questions.

ACKNOWLEDGMENTS

We thank D. Bohmann and S. Lippard for mammalian epitope-tagged ubiquitin expression plasmids, S. Kornbluth for pcDNA/BAT3::Myc, and J. P. Vogel for pJB3395. We thank H. Steen and the Proteomics Center at Children's Hospital Boston for protein identification by mass spectrometry. We thank the members of the Isberg laboratory for critical review of the manuscript.

A.W.E. was supported by a postdoctoral Ruth L. Kirschstein National Research Service Award from the National Institutes of Health. R.R.I. is a Howard Hughes Medical Institute investigator.

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Editor: R. P. Morrison